

**Genetic variants of the human FSH receptor and the influence thereof on
gametogenesis**

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The present invention provides a method for determining the dosage of FSH in the treatment of infertility of women comprising the determination of the FSH receptor variant of the woman to be treated, a method for treating infertility in women which comprises said determination of the FSH receptor variant and a kit for performing said determination of the FSH receptor variant.

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The success of controlled ovulation induction in the course of an assisted reproduction procedure depends on the administration of the hormone FSH. Unfortunately neither the patients's reaction towards the administration of FSH nor which hormone dose is necessary can be foreseen at the beginning of the treatment phase. Due to the lack of predictive parameters a high number of expensive ampoules of FSH is being used in IVF clinics for the induction of ovulation, a treatment regimen that bears the danger of overstimulation and its clinical consequences.

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The follicle-stimulating hormone (FSH) is an essential factor for the maturation of germ cells (gametogenesis) in both men and women. FSH exerts its action via the FSH receptor which is specifically located in the granulosa cells in the ovary and in the Sertoli cells in the testis. Any perturbation of the interaction between FSH and its receptor leads to impairment of gametogenesis. Women with FSH receptor mutations show a clinical picture typical of primary amenorrhoea, men with FSH receptor mutation are generally subfertile. These observations underline the central role of FSH and its receptor for a normal physiological maturation of oocytes as well as spermatozoa (Nieschlag et al., Clin. Endocrinol. 51:139-146 (1999)).

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The FSH receptor is present on the cell membrane and consists of an extracellular, a transmembrane and an intracellular domain. The FSH receptor gene is located on chromosome 2p21 and consists of 10 exons. Exons 1 to 9 encode for the

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extracellular domain while exon 10 encodes for the transmembrane and intracellular domain. The whole gene spans a region of over 54 kbp encoding a mature protein of 695 amino acids (Gromoll et al. Genomics 35, 308-311 (1996)).

- 5 Our recent studies have shown that the FSH receptor exists in two genetic variants. Amino acid position 307 is either occupied by alanine or threonine and at position 680 either serine or asparagine is found. The amino acid position 307 is located in the extracellular domain while the amino acids at position 680 are part of the intra-cellular domain. We have shown that both positions are genetically linked with each other. Due to that two discrete FSH receptor variants can be usually found consisting of either threonine 307 and asparagine 680 or alanine 307 and serine 680 (Fig. 1). Both receptor variants are statistically equally distributed. Further, Simoni et al., J. Clin. Endocrinol. Metab. 84:751-755 (1999) and Conway et al., Clinical Endocrinology, vol. 51, 97-99 (1999) describes that the FSH receptor variants can be analysed by restriction enzyme analysis.

- 15 So far functional studies could not show any significant differences as to hormone binding or signal transduction between the two receptor variants. It should be noted, however, that the model system used so far for functional studies is not sensitive enough to detect subtle differences in FSH-FSH receptor interaction and receptor activation. In a first clinical study comparing infertile men with fertile men no significant differences of receptor variant distribution could be detected (Simoni et al., J. Clin. Endocrinol. Metab. 84:751-755 (1999)).

- 25 In an additional study completed recently we have investigated normal women who were treated with FSH in the course of an assisted reproduction procedure. The administration of FSH leads to a controlled induction of ovulation which makes it possible to gain oocytes from the patient. The oocytes are then incubated with sperm in vitro and the nascent zygotes are re-implanted into the patient's uterus. The aim of the FSH treatment is to gain a sufficient number of oocytes capable of being fertilized. Clinical experience shows, however, that patients react differently towards the stimulation with FSH. While some patients need relatively low doses of FSH in order

to produce a sufficient number of oocytes, other patients have to be stimulated with high doses of FSH to reach the same results in terms of a sufficient number of oocytes. Depending on the amount of FSH necessary the patients can be classified into good responders (low dose of FSH necessary) and bad responders (high dose of FSH necessary). The reasons for the difference in sensitivity towards stimulation with FSH are so far not known. The need for adjusting the FSH dose over the course of time during the stimulation phase in order to give rise to a sufficient number of oocytes without provoking an overstimulation represents a major problem in IVF treatment. The clinical picture of differences in the sensitivity towards FSH in patients undergoing assisted reproduction procedure was the starting point for our investigation in which we tested the hypothesis that different FSH receptor variants are responsible for the differences in FSH sensitivity.

We have screened 160 patients from our IVF department and could show that patients bearing the homozygous FSH receptor variant alanine 307/serine 680 need significantly more FSH for the stimulation of oocyte maturation than the patients with the homozygous FSH receptor variant threonine 307/asparagine 680. It became further obvious that in patients with a heterozygous state of FSH receptor variants an intermediate dose of FSH was necessary for ovulation induction (Fig. 2). Moreover, the receptor variants seem to regulate the basal serum levels of FSH since patients bearing the homozygous FSH receptor variant alanine 307/serine 680 show a mean basal FSH level of 7.9 IU/l, while in patients with a heterozygous receptor variant and in patients homozygous for the FSH receptor variant threonine 307/asparagine 680 the mean basal FSH level is 7.0 IU/l and 6.3 IU/l, respectively (Fig. 3).

Thus, it was found that the response of patients towards the stimulation with FSH is depending on the allelic variant of the FSH receptor. In order to determine which particular variant of the FSH receptor is present in a given patient a simple analysis of DNA extracted from blood cells has to be conducted. The individual amount of FSH to be administered could be determined according to which FSH receptor variant the patient possesses. Due to these findings it can be expected that in the future the genetic analysis of the FSH receptor region determining the variant will play an

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important role for the planning of ovulation induction treatment. In other words, it was found that the FSH receptor variants determine differences in sensitivity towards FSH. This finding has a great impact on the FSH therapy in the course of an assisted reproduction procedure since it makes a predetermined and individually adjusted FSH stimulation protocol possible depending on the FSH receptor variant present in a given patient. The potential benefits of such an adjusted FSH stimulation therapy are profound. This approach will not only improve the clinical safety, by avoiding dangerous overstimulation with FSH, but will also help to reduce treatment costs significantly, since FSH is quite expensive.

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Finally, FSH receptor variants may be associated with reduced fertility in men and women and that the analysis of such variants may have great impact on the treatment of reduced fertility with FSH.

15 The invention thus provides

(1) a method for determining, i.e., predicting the dosage of follicle-stimulating hormone (FSH) in the treatment of infertility of women comprising the determination of the FSH receptor variant of the woman to be treated;

20 (2) a method for treating infertility in women which comprises determining the FSH receptor variant in the woman to be treated as defined in (1) above;

(3) a kit for performing the determination of the FSH receptor variant in a woman as defined in (1) and (2) above; and

(4) a FSH preparation comprising a specific amount of FSH which is suitable as a daily dosage for high dosage, intermediate dosage or low dosage FSH treatment.

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The attached Figures 1 to 6 depict the following:

Fig. 1 shows the two FSH receptor variants.

30 Fig. 2 shows the number of FSH ampoules (75 IU/ampoule) required to achieve ovulation induction and oocyte retrieval in normoovulatory women in an assisted reproduction programme.

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(data: mean \pm SEM)

* $p < 0.05$ vs A/A (Kruskal Wallis test)

Fig. 3 shows the basal FSH levels (day 3) in normoovulatory women grouped according to the FSH receptor genotype.

(data: mean \pm SEM)

* $p < 0.05$ vs A/A (Kruskal Wallis test)

Fig. 4 shows the restriction fragment length polymorphism of Asn 680 Ser of the FSH receptor.

Fig. 5 shows the DNA sequence of exon 10 of the FSH receptor gene (EMBL accession No. X91747).

Fig. 6 shows exon 10 of the FSH receptor. Suitable primers are underlined. The reverse primers A₂-G₂ are complementary to the respective underlined sequences in this figure.

The method (1) of the invention is hereinafter described in more detail. First of all, the determination of the FSH receptor variant is preferably performed *in vitro*.

Secondly, since the polymorphic sites are genetically coupled in the FSH receptor variants, the sequence analysis of one variant site is sufficient. It is preferred that the analysis is performed by the "restriction fragment length polymorphism" (RFLP) method. The first step of said method is the isolation of genomic DNA from a patient's blood sample and the amplification of a specific part of the FSH receptor by PCR. The amplicon obtained in the PCR is cut with a restriction enzyme which specifically recognizes the amino acid sequence at position 680, e.g. Bsr I. A complete restriction of the amplicon indicates the presence of a homozygous serine, in the case of an incomplete restriction a heterozygous receptor status is present and no restriction of the amplicon indicates a homozygous asparagine (Fig. 4). Suitable primers for the PCR reaction are shown in Fig. 6.

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The determination of the variant may also be performed by the "single stranded conformation polymorphism" (SSCP) method and/or by the "allele specific amplification" method. The RFLP, SSCP and "allele specific amplification" methods are generally known in the art (e.g. from Oldenburg, M.C. and Siebert, M., New Cleavage Fragment Length Polymorphism method improves the mutation detection assay, Biotechniques, Feb. (2000), 28(2):351 and Shi, M. M. et al., Technologies for detecting genetic polymorphisms in pharmacogenomics, Mol. Diagn. Dec. (1999), 4(4):343-51), which we hereby incorporate by reference.

The detection of a single nucleotide change leading to an amino acid exchange is ideally suited for the development of a specific kit which would make the detection of the FSH receptor variants easy and fast. Such a kit would ideally be used for the screening of patients prior to a FSH therapy.

In the FSH therapy and after determination of the FSH receptor, the women bearing the homozygous FSH receptor variant Ala307/Ser680 may be given a high dosage of FSH, namely about 42-48 ampoules FSH within a treatment period of 14 days, which corresponds to a daily dosage of greater than 225, preferably 230 to 250 International Units (IU) FSH; the women bearing the homozygous FSH receptor variant Thr307/Asn680 may be given a low dosage of FSH, namely 30-35 ampoules FSH per 14 days, which corresponds to a daily dosage of 150 ± 20 IU FSH; and the women with a heterozygous state may be given an intermediate dosage of FSH, namely about 36 to 41 ampoules per day, which corresponds to a daily dosage of 200 ± 20 IU FSH. The FSH is preferably administered subcutaneously.

The FSH preparation of embodiment (4) of the invention contains the high dosage, intermediate dosage or a low dosage FSH as set forth in detail above. The preparation is preferably in an injectable form and may contain suitable additives (such as buffers, saline, etc.) known in the art.

The invention is further illustrated by the following non-limitative example.

Example 1: Determination of the restriction fragment length polymorphism (Asn680Ser, hFSH receptor)

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A. PCR: Amplification with primers E₁ and G₂ (Exon 10 of the FSH receptor)

For each sample is pipetted:

- 36 µl autoclaved distilled water
- 10 5 µl Thermo-Buffer 10x (Promega)
- 3 µl MgCl₂ (25 mM, Promega)
- 2.5 µl dNTP-solution (1mM, Pharmacia)
- 1 µl Primer E₁ (0.1 µg/µl; 5'-CCTTGTGCTAATGTCCTGG)
- 1 µl Primer G₂ (0.1 µg/µl; 5'-TGTAGAAGCACTGTCAGCTC)
- 15 0.5 µl Taq DNA polymerase (5000 IU/ml, Promega)
- 1 µl DNA (DNA extracted from 5-10 ml and dissolved in 50 µl distilled water)

PCR program:

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|----|------|--------|-----------|
| 20 | 94°C | 4 min | 1 cycle |
| | 94°C | 1 min | |
| | 58°C | 30 s | 35 cycles |
| | 72°C | 50 s | |
| 25 | 72°C | 10 min | 1 cycle |
| | 30°C | 30 min | |

The PCR product is checked on a 2% TAE agarose gel. The size of the desired band is 580 kbp.

- 30 Subsequently a phenol-chloroform cleaning is performed twice, the resulting DNA is precipitated with 0.5 sample volumes 7.5 M ammonium acetate and 2.5 volumes ab-

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solute ethanol, washed with 70% ethanol and air-dried. Finally, the DNA is re-dissolved in 17 µl water.

B. Digestion:

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2 µl buffer NEB 3 10x (Biolabs) and

1 µl Bsr I (Biolabs) are added to the sample, the resulting mixture is overlayed with 2 drops of mineral oil and digested for 1.5 hours at 65°C. The digestion is checked on a 2-2.5% TAE agarose gel.

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The enzyme Bsr I has a restriction site for TGACC. If the FSH receptor contains the amino acid serin at position 680 of the 5th transmembrane domain, Bsr I cuts the PCR product in two bands (443 and 136 bp). The enzyme cannot digest the PCR product if the amino acid at position 680 is asparagin. The single band on the gel has a size of 579 bp (see Fig. 4).

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C. Results:

	One band size 579 bp	→	asparagin, homozygous
20	Two band sizes 443 and 136 bp	→	serin, homozygous
	Three band sizes 579, 443 and 136 bp	→	asparagin/serin heterozygous

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Example 2: We started a prospective study which women were screened for the FSH receptor variant before starting the FSH treatment. Only women with homozygous FSH receptor variant at position 680 were included in the study and were randomised to receive a pre-determined, fixed dosage of FSH. The preliminary results, based on 32 cycles, confirm that the Ser 680 variant is less sensitive to FSH stimulation (in terms of production of estradiol) and that more FSH is necessary to induce the degree of stimulation observed in women with the Asn 680 variant.

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These results reinforce the idea that the analysis of the FSH receptor variant is useful for the determination of the FSH dosage.

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